Award Number: DAMD17-01-1-0273

TITLE: Breast Tumor Kinase Signaling in Breast Cancer

PRINCIPAL INVESTIGATOR: Angela L. Tyner, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, Illinois 60612-7227

REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (1 Jun 02 - 31 May 03)

4. TITLE AND SUBTITLE
Breast Tumor Kinase Signaling in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0273

6. AUTHOR(S)
Angela L. Tyner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois at Chicago
Chicago, Illinois 60612-7227

E-Mail: atyner@uic.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We previously demonstrated that the breast tumor kinase Brk can phosphorylate and inhibit the RNA-binding protein Sam68, which plays important roles in RNA metabolism associated with growth. Two novel Sam68-like mammalian proteins SLM1 and SLM2 were identified, and cotransfection of NMuMG cells with different Brk expression constructs and either GFP-SLM1 or GFP-SLM2 revealed a direct correlation between Brk activity and the level of SLM1 and SLM2 phosphorylation. Mutation of the terminal tyrosine in Brk (Brk Y-F) increased the level of SLM protein phosphorylation. Localization of tyrosine phosphorylated proteins was examined in NMuMG cells cotransfected with Brk constructs and GFP-tagged substrates. Interestingly phosphotyrosine immunoreactivity was restricted to the nucleus and co-localized with the GFP-tagged nuclear substrates only when Brk Y-F was expressed. Expression of wildtype Brk resulted in phosphorylation of cytoplasmic and nuclear proteins. This suggests a role for the carboxy terminal tyrosine in localization of Brk. Fractionation of cells transfected with Brk expression constructs showed that Brk (Y-F) was enriched in the nuclear fraction. These data support our hypothesis that the RNA binding proteins SLM1 and SLM2 are substrates of Brk. Brk may regulate posttranscriptional control of gene expression by modifying nuclear activities of Sam68 and related proteins such as SLM1 and SLM2.

14. SUBJECT TERMS

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....6

Reportable Outcomes.....12

Conclusions.....13

References.....14

Appendices.....

Introduction

The intracellular breast tumor kinase Brk (also called Sik or PTK6) was identified in a human metastatic breast tumor in a screen for protein tyrosine kinases involved in breast cancer [1], from the mouse small intestine in a screen for factors that regulate epithelial cell differentiation [2], and from cultured human melanocytes [3]. Brk is expressed in many breast carcinoma cell lines and in a high percentage of primary breast tumors that have been examined, but it has not been detected in normal human breast tissue [1, 4], or at any stage of mammary gland differentiation in the mouse [5]. Brk is expressed in skin and throughout the human gastrointestinal tract and moderate increases in Brk levels were detected in colon tumors [5]. In healthy epithelial linings, expression of Brk correlates with cell differentiation.

Substrates identified for Brk include the novel adaptor-like protein BKS [6] and the nuclear RNA-binding protein Sam68 [7]. Sam68 is a member of the STAR family of KH-domain containing RNA-binding proteins that regulate RNA metabolism [8]. In cancer cell lines Sam68 is localized to unique nuclear structures termed Sam68/SLM nuclear bodies (SNBs) [9]. After stress such as heat shock, Sam68 can also be found in stressed induced nuclear bodies [10]. Although Sam68 can be phosphorylated by other intracellular tyrosine kinases, only Brk has been shown to colocalize with Sam68 in the nucleus [7].

Several studies indicate that Sam68 regulates RNA metabolism. Sam68 was found to act as a functional homologue of the human immunodeficiency virus type 1 (HIV1) Rev protein, which transports RNA from the nucleus to the cytoplasm [11, 12]. We showed that Brk phosphorylation of Sam68 inhibits its ability to bind RNA and to function as a cellular Rev homologue [7]. Sam68 has been shown to influence the utilization of specific RNAs in the cytoplasm and this is also functionally regulated by Brk [13]. In addition, Sam68 co-localizes with and associates with RNA splicing factors [10, 14], and was recently shown to be a regulator of alternative splicing [15].

The ability of Brk to negatively regulate the RNA-binding activities of Sam68 may have an impact on cell growth and differentiation. It has been suggested that RNA-binding functions of Sam68 positively regulate cell growth because a variant of Sam68 lacking a functional KH domain inhibits cell cycle progression [16]. Sam68 is also a direct target of Cdc2 during mitosis [17] and an extracellular signal-regulated kinase (ERK) target [15].

While localized in the nucleus, Brk can phosphorylate and inhibit Sam68. However, when Brk is excluded from the nucleus it may facilitate signaling by the ErbB family of receptor tyrosine kinases. Ectopic expression of Brk sensitized the mammary epithelial cells to epidermal growth factor (EGF), and improved their growth in response to EGF stimulation [18]. Brk appeared to associate with the EGF receptor (ErbB1) in mammary epithelial cells in coimmunoprecipitation experiments, although the tyrosine phosphorylation of ErbB1 was not altered in cells overexpressing Brk [18]. Brk expression in mammary epithelial cells also enhanced tyrosine phosphorylation of the ErbB3 receptor when cells were treated with EGF [19].

Recently, two Sam68-like-mammalian proteins, SLM-1 and SLM-2 were identified [20]. SLM-1 and SLM-2 have characteristic Sam68 SH3 domain binding sites. In addition, ectopically expressed SLM-1 and SLM-2 colocalize with endogenous Sam68 in Sam68 nuclear bodies (SNBs) in cancer cell lines [9]. The roles of Sam68 and the related proteins SLM1 and SLM2 have not been studied in breast cancer. Since Brk is aberrantly expressed in Brk cancers, we hypothesized that inappropriate phosphorylation of Brk substrates such as Sam68, SLM1 and SLM2 may contribute to the development of breast cancer.

BODY

The goal of our grant is to characterize RNA-binding protein substrates of Brk and to determine the biological significance of phosphorylation of these substrates in breast cancer. During the past year of funding we have determined that wildtype Brk and Brk with a mutation of its carboxy terminal tyrosine Y447 to F (Brk Y-F) phosphorylate SLM-1 and SLM-2 in mammary gland epithelial cells. We found that Brk Y-F is much more effective at phosphorylating all of its STAR family protein substrates. It was demonstrated that mutation of this C-terminal tyrosine increases enzyme activity and SH2 domain accessibility, consistent with a role for the carboxy terminal tyrosine in autoinhibition [7, 21]. We examined localization of the transfected proteins using confocal microscopy and we discovered that the mutated activated form of Brk is more concentrated in the nucleus, which may also enhance its ability to phosphorylate nuclear substrates. We determined that there are a number of possible additional substrates for Brk that co-immunoprecipitate with activated Brk, in mammary epithelial cells. In addition to Sam68, several unidentified tyrosine phosphorylated proteins also associate with Brk.

Work proposed in the Idea grant *(taken from technical abstract of funded proposal)*

We hypothesize that induction of Brk in breast tumors leads to modification of STAR family proteins resulting in changes in gene expression that contribute to development of breast cancer. To test this, we will examine expression and phosphorylation of STAR protein substrates of Brk in normal and cancerous breast cells (Aims 1 and 2). Then we will determine if altering Brk activity results in changes in RNA or protein expression, and if these changes require functional STAR proteins (Aim 3). The proposed experiments will enhance our understanding of Brk signaling and the STAR proteins in breast tumors. Brk and/or factors regulated by the Brk signaling pathway may provide therapeutic targets for the treatment of breast cancer.

Key Research Accomplishments

Using cotransfection, immunoprecipitation, and immunoblotting, we have determined that Brk efficiently phosphorylates SLM-1 and SLM-2 in mammary gland epithelial cells. Following transfection of normal murine mammary gland epithelial cells with either wildtype Brk and Brk Y-F, and GFP-tagged-SLM1 and GFP-SLM2, the phosphorylation status of the proteins was determined following SDS-PAGE and immunoblotting using anti-phosphotyrosine antibodies **(Figure 1)**. The GFP-tagged proteins are clearly phosphorylated when coexpressed with Brk. Of note, the Brk(Y-F) construct with mutation of the terminal tyrosine 447 is more effective.

Using confocal microscopy, we examined localization of transfected proteins and protein tyrosine phosphorylation using antibodies specific for GFP, anti-phosphotyrosine, and Brk **(Figures 2 and 3)**. The RNA-binding proteins Sam68, SLM1, and SLM2 always show nuclear-specific localization. Interestingly, following transfection of wildtype Brk and Brk (Y-F), we noticed differences in the intracellular localization of phospho-tyrosine specific immunoreactivity. Transfection of wildtype Brk leads to phosphorylation of cytoplasmic and nuclear proteins, while the Brk Y-F construct is more nuclear specific and phospho-tyrosine immunoreactivity is concentrated in the nucleus. An example of the differences in localization of wildtype Brk and Brk Y-F activity is shown for a series of transfection experiments with GFP-tagged SLM2 **(Figure 2).**

To further examine differential intracellular localization of Brk, NMuMG cells were transfected with wildtype Brk, kinase defective Brk (Brk K-M) and Brk Y-F expression constructs, and cells were fractionated and proteins were isolated from nuclear versus

membrane/cytoplasm fractions. Immunoblotting with Brk antibodies demonstrated that Brk Y-F is more concentrated in the nucleus than wildtype Brk. Thus Brk Y-F has greater access to its nuclear substrates and may be retained in the nucleus through SH2/SH3 domain mediated interactions **(Figure 3)**.

To study Brk regulation of gene expression in mammary gland epithelial cells, we generated stable cell lines expressing the different Brk expression constructs. However, kinase activity of Brk in these cell lines is repressed. In transient transfection assays, we find peak Brk kinase activity at 24 hours post transfection. By 48 hours, Brk kinase activity levels are greatly reduced although protein expression levels increase. This indicates that mammary gland epithelial cells have a mechanism for inhibiting Brk kinase activity, which may be disrupted in breast cancer. Current efforts are directed at development of inducible expression systems. We are also performing transient transfection studies with Sam68 alone and in combination with the various Brk expression constructs, and examining patterns of gene and protein expression. Following transfection of NMuMG cells with Brk expression constructs we detect phosphorylation of a number of proteins **(Figure 4)**. Wildtype Brk and Brk Y-F phosphorylate endogenous Sam68 in NMuMG cells. In addition there are several novel proteins that are phosphorylated, and many of these coimmunoprecipitate with Brk. A goal of the coming year will be to identify some of these novel substrates and to determine if Sam68 modulates their expression.

FIGURE 1

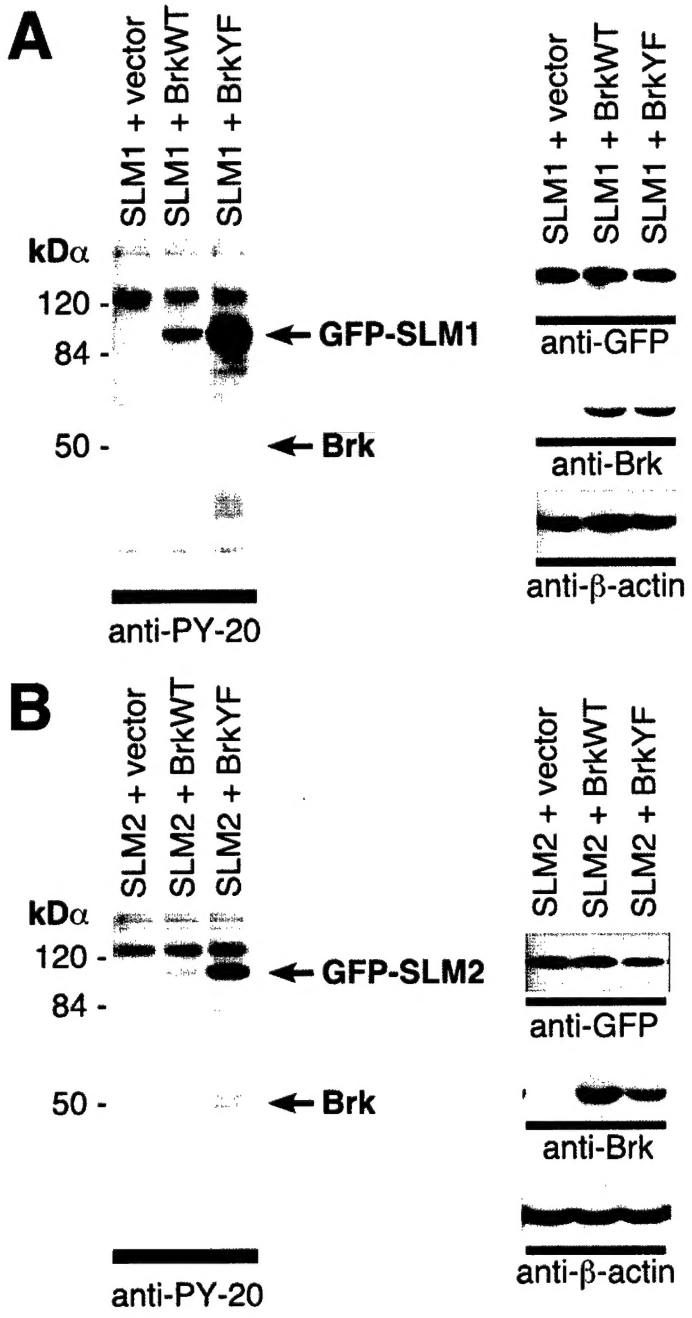

Figure 1. Brk phosphorylates the STAR Proteins SLM-1 and SLM-2 in NMuMG cells. GFP-tagged SLM1 **(A)** or SLM2 **(B)** was cotransfected with wildtype Brk, Brk Y-F or empty expression vector. Total cell lysates were resolved by SDS PAGE and immunoblotting experiments were performed with the indicated antibodies. Tyrosine phosphorylation of GFP-tagged SLM1 and SLM2 is detectable with anti-phosphotyrosine antibodies (anti-PY-20) in cells expressing Brk. Controls examining expression of the transfected proteins are shown at the right.

FIGURE 2

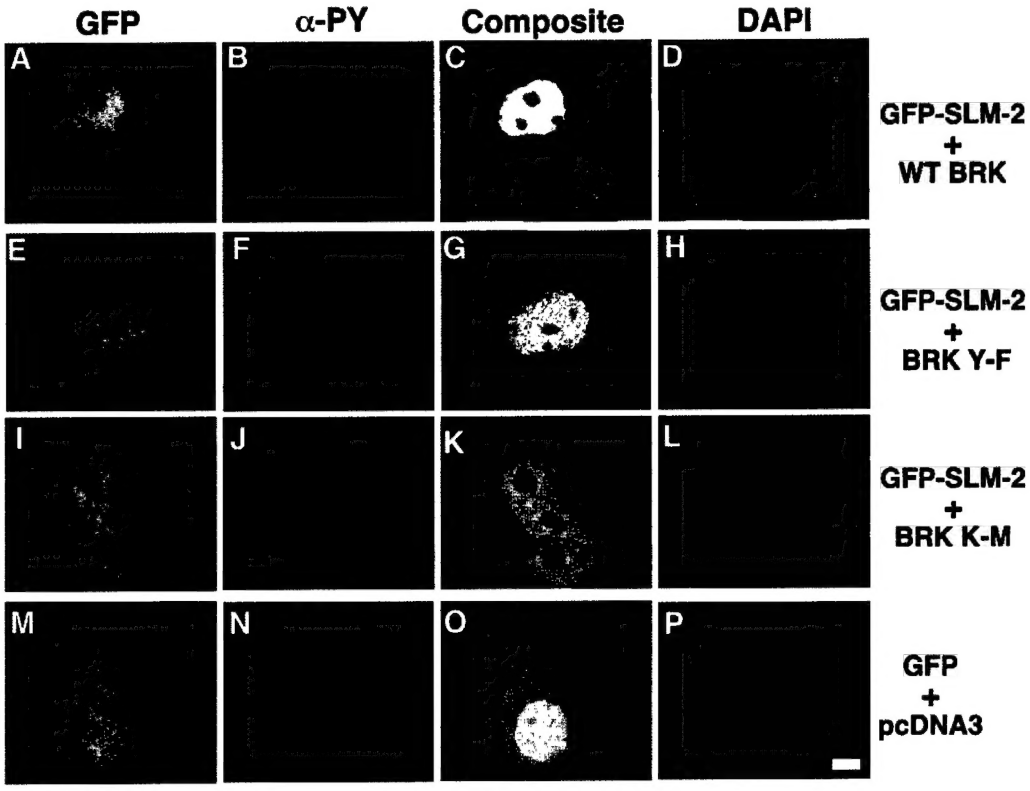

Figure 2. Wildtype Brk and Brk Y-F phosphorylate nuclear proteins that colocalize with GFP-SLM2 within the nucleus.

NMuMG cells were transfected with GFP-SLM2 and wildtype Brk (A-D), GFP-SLM2 and Brk Y-F (E-H), GFP-SLM2 and kinase defective Brk K-M (I-L), or the GFP expression vector pEGFP-C1 and pcDNA3 (M-P). Cells were fixed 24 hours after transfection and tyrosine phosphorylated proteins were localized using anti-phosphotyrosine antibodies (B, F, J , N). DAPI was used to stain the nuclei (D, H, L, P). In NMuMG cells, SLM2 displays diffuse, nuclear localization visible by green fluorescence (A, E, I). Cells cotransfected with GFP-SLM2 and wild type Brk or Brk Y-F also stain strongly with the anti-phosphotyrosine antibody visualized using rhodamine (B, F), while no phosphotyrosine is detected in cells expressing kinase defective Brk K-M (J). Interestingly, phosphotyrosine immunoreactivity is seen in the cytoplasm and at the membrane when wildtype Brk is transfected into the cells, but only in the nucleus following transfection of Brk Y-F. Panels C, G, K, and O are composites demonstrating colocalization that appears yellow. GFP alone is expressed throughout the cell (M), and is negative for anti-phosphotyrosine staining (N). Bars represent 5 micrometers.

FIGURE 3

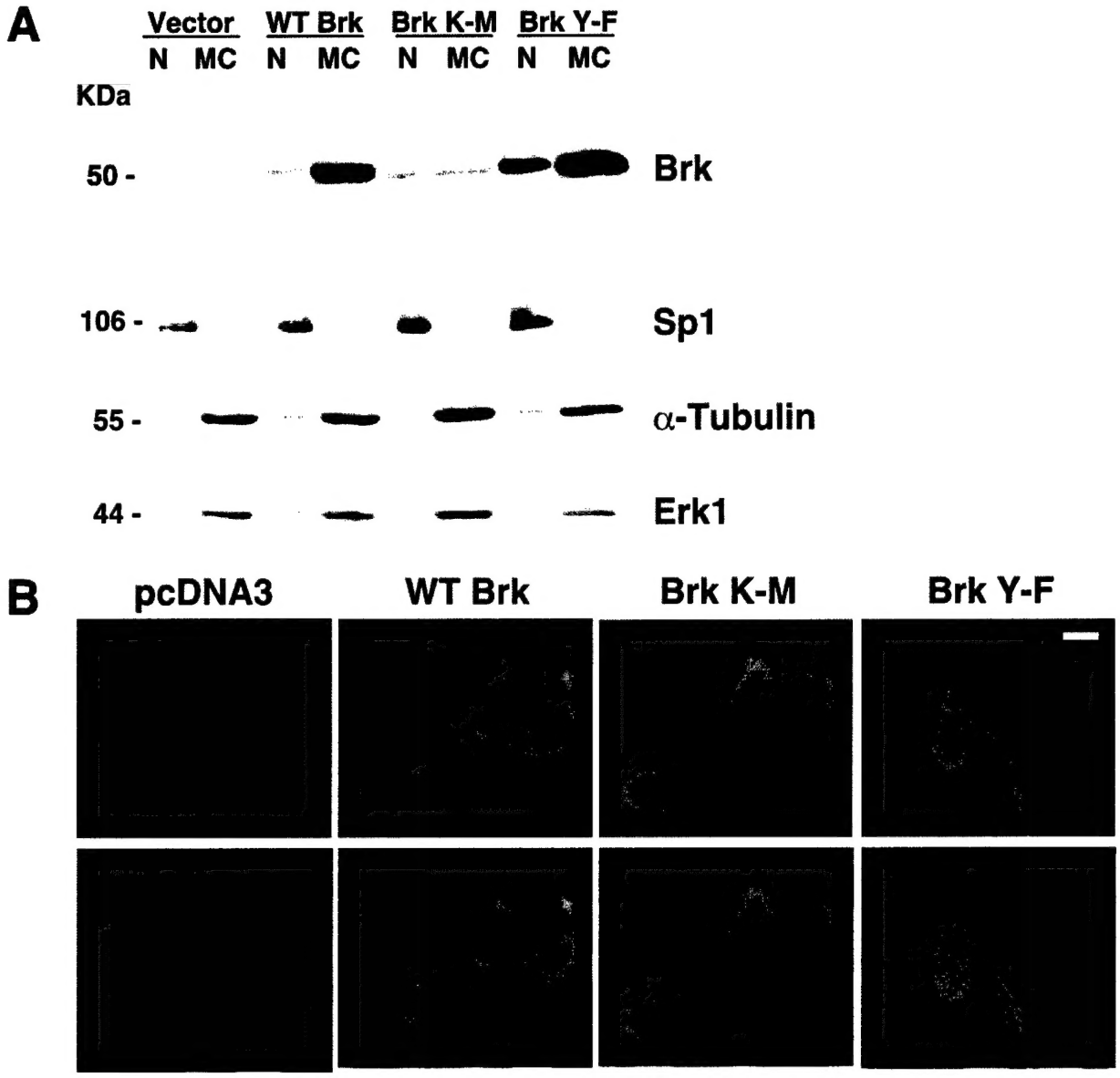

Figure 3. Intracellular localization of wildtype (WT), kinase defective (K-M) and activated (Y-F) Brk in transfected NMuMG cells. A) Transfected NMuMG cells were fractionated into nuclear and membrane/cytoplasmic fractions and localization of transfected Brk protein was examined by immunoblotting. A greater proportion of Brk Y-F is found in the nucleus. B) Localization of transfected Brk protein was examined using immunohistochemistry and confocal microscopy. Brk Y-F exhibits increased nuclear localization.

FIGURE 4

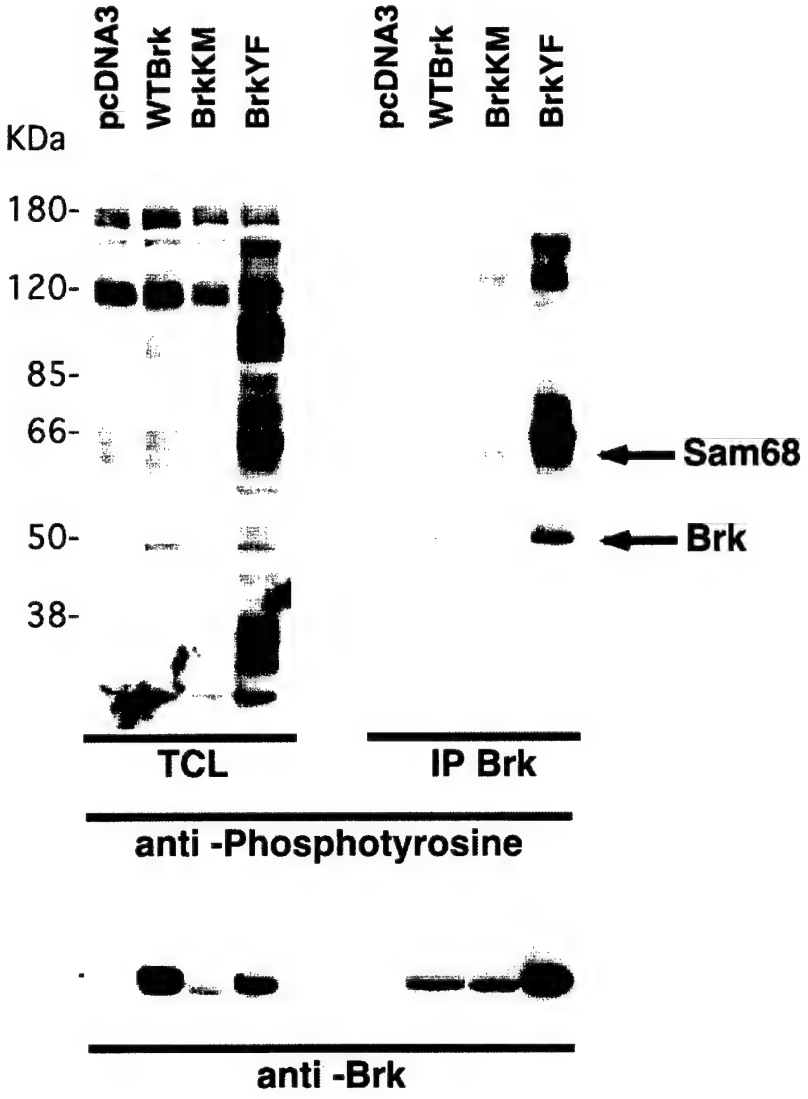

Figure 4. Phosphorylation of proteins by Brk in NMuMG cells. Empty vector (pcDNA3), wildtype (WT), kinase defective (KM) and activated (YF) Brk were transfected into NMuMG cells. Total cell lysates were isolated and phosphorylation of proteins following introduction of Brk was examined by immunoblotting with antibodies against phosphotyrosine. Transfected Brk protein was also immunoprecipitated and tyrosine phosphorylated Brk associated proteins were visualized. Sam68 is one of the proteins that is tyrosine phosphorylated by Brk and coimmunoprecipitates with Brk. However other potential substrates and associated proteins remain unidentified.

Reportable Outcomes

During the second year of funding we have further examined the abilities of Brk to regulate STAR family RNA-binding proteins. Brk is able to phosphorylate Sam68, SLM1 and SLM2 in mammary gland epithelial cells. We found that mutation of the carboxy terminal tyrosine results in increased nuclear localization of Brk, which may contribute to its increased ability to phosphorylate SLM proteins. During the first year of funding we determined that only Sam68 is expressed at significant levels in normal mammary gland and in breast tumor cell lines (AIM 1). SLM1 and SLM2 have much more restricted patterns of gene expression than Sam68, and SLM2 appears to be testes-specific. We have made progress in examining expression of other Brk regulated proteins in mammary gland epithelial cells. Phosphorylation of a number of proteins increases upon overexpression of activated Brk Y-F and efforts are underway to identify proteins that co-immunoprecipitate with Brk.

A manuscript describing results of our studies addressing experiments outlined in Aim 1 and 3 is in preparation and will be submitted shortly.

Darien Heap, Andrea Haegebarth, Wenjun Bie, Jason J Derry, Stephane Richard, and Angela L. Tyner. 2003. The Sam68-like mammalian proteins SLM1 and SLM2 are nuclear substrates of the Brk/Sik tyrosine kinase. In preparation.

In related studies, a manuscript describing the functions of Brk/Sik and related kinases in growth control has been published.

Serfas M.S. and A. L. Tyner. 2003. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. Oncology Res. 13, 409-419.

The following abstract was presented at the 2002 ERA OF HOPE Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting in Orlando, Florida:

Angela L. Tyner, Stephane Richard, Jason J. Derry, Xin Ye, and Wenjun Bie. The breast tumor kinase Brk is a nuclear tyrosine kinase that phosphorylates and regulates the RNA-binding protein Sam68 and the Sam68-like mammalian proteins SLM-1 and SLM-2

Conclusions

We are investigating the role of Brk in the etiology of breast cancer. Over the past year we have made the important finding that intracellular localization may be an important regulator of Brk signaling. Brk is not myristoylated and not targeted to the membrane like members of the Src family. In transfection experiments we found that Brk efficiently enters the nucleus where it can phosphorylate and inhibit RNA-binding proteins of the STAR family. Over the last year we have determined that mutation of the carboxy-terminal tyrosine of Brk not only prevents negative regulation of the enzyme by tyrosine phosphorylation, but may also alter intracellular localization of a pool of the protein. Intracellular localization of Brk in breast cancers may influence its ability to regulate tumor development.

We have completed specific aim 1 and are currently completing experiments outlined in aims 2 and 3 of the original proposal, with a focus on Sam68, the only STAR protein that we found to be expressed at significant levels in mammary gland epithelial cells.

ABBREVIATIONS

Brk: **Br**east Tumor **K**inase

NMuMG: **N**ormal **Mu**rine **M**ammary **G**land

Sam68: **S**rc-**a**ssociated in **m**itosis, **68** kDa

Sik: **S**rc-related **i**ntestinal **k**inase (original name for mouse Brk)

SLM-1: **S**am68-like-**m**ammalian protein-1

SLM-2: **S**am68-like-**m**ammalian protein-2

SNBs: **S**am68/SLM **N**uclear **B**odies

STAR: **S**ignal **T**ransducers and **A**ctivators of **R**NA

REFERENCES

1. Mitchell, P.J., et al., *Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours.* Oncogene, 1994. **9**: p. 2383-2390.
2. Siyanova, E.Y., et al., *Tyrosine kinase gene expression in the mouse small intestine.* Oncogene, 1994. **9**(7): p. 2053-7.
3. Lee, S.T., K.M. Strunk, and R.A. Spritz, *A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes.* Oncogene, 1993. **8**(12): p. 3403-10.
4. Barker, K.T., L.E. Jackson, and M.R. Crompton, *BRK tyrosine kinase expression in a high proportion of human breast carcinomas.* Oncogene, 1997. **15**(7): p. 799-805.
5. Llor, X., et al., *BRK/Sik expression in the gastrointestinal tract and in colon tumors.* Clin Cancer Res, 1999. **5**(7): p. 1767-77.
6. Mitchell, P.J., E.A. Sara, and M.R. Crompton, *A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK.* Oncogene, 2000. **19**(37): p. 4273-82.
7. Derry, J.J., et al., *Sik (BRK) Phosphorylates Sam68 in the Nucleus and Negatively Regulates Its RNA Binding Ability.* Mol Cell Biol, 2000. **20**(16): p. 6114-6126.
8. Vernet, C. and K. Artzt, *STAR, a gene family involved in signal transduction and activation of RNA.* Trends Genet, 1997. **13**(12): p. 479-84.
9. Chen, T., et al., *A Role for the GSG Domain in Localizing Sam68 to Novel Nuclear Structures in Cancer Cell Lines.* Mol Biol Cell, 1999. **10**(9): p. 3015-3033.
10. Denegri, M., et al., *Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors.* Mol Biol Cell, 2001. **12**(11): p. 3502-14.
11. Reddy, T.R., et al., *Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev.* Nat Med, 1999. **5**(6): p. 635-42.
12. Soros, V.B., et al., *Inhibition of human immunodeficiency virus type 1 Rev function by a dominant-negative mutant of Sam68 through sequestration of unspliced RNA at perinuclear bundles.* J Virol, 2001. **75**(17): p. 8203-15.
13. Coyle, J.H., et al., *Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK.* Mol Cell Biol, 2003. **23**(1): p. 92-103.
14. Hartmann, A.M., et al., *The interaction and colocalization of Sam68 with the splicing-associated factor YT521-B in nuclear dots is regulated by the Src family kinase p59(fyn).* Mol Biol Cell, 1999. **10**(11): p. 3909-26.
15. Matter, N., P. Herrlich, and H. Konig, *Signal-dependent regulation of splicing via phosphorylation of Sam68.* Nature, 2002. **420**(6916): p. 691-5.
16. Barlat, I., et al., *A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain.* J Biol Chem, 1997. **272**(6): p. 3129-32.
17. Resnick, R.J., et al., *Phosphorylation of the Src substrate Sam68 by Cdc2 during mitosis.* Oncogene, 1997. **15**(11): p. 1247-53.
18. Kamalati, T., et al., *Brk, a breast tumor-derived non-receptor protein-tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor.* J Biol Chem, 1996. **271**(48): p. 30956-63.

19. Kamalati, T., et al., *Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation.* Oncogene, 2000. **19**(48): p. 5471-6.
20. Di Fruscio, M., T. Chen, and S. Richard, *Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM- 1 is a Src substrate during mitosis.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2710-5.
21. Qiu, H. and W.T. Miller, *Regulation of the nonreceptor tyrosine kinase Brk by autophosphorylation and by autoinhibition.* J Biol Chem, 2002. **277**(37): p. 34634-41.

Personnel Receiving Support From the Idea Award

P.I.	Angela L. Tyner, Ph.D.
Research Technician (100%)	Wenjun Bie, M.S.
Research Assistant (100%)	Andrea Haegebarth, M.S.